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Increased Vascular Permeability measured with an Albumin-Binding MR Contrast Agent is a Surrogate Marker of Rupture-Prone Atherosclerotic Plaque

Running title: MRI of vascular permeability and plaque instability

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ABSTRACT

Background – Compromised structural integrity of the endothelium and higher microvessel density increase vascular permeability. We investigated whether vascular permeability measured *in vivo* by magnetic resonance imaging (MRI) using the albumin-binding contrast agent, gadofosveset, is a surrogate marker of rupture-prone atherosclerotic plaque in a rabbit model.

Methods and Results – New Zealand White rabbits (n=10) were rendered atherosclerotic by cholesterol-diet and endothelial denudation. Plaque rupture was triggered with Russell's viper venom and histamine. Animals were imaged before (pre) triggering, at 3 and 12-weeks, to quantify plaque area, vascular permeability, vasodilation, and stiffness and post-triggering to identify thrombus. Plaques identified on the pre-trigger scans were classified as stable or rupture-prone based on the absence or presence of thrombus on the corresponding post-trigger MRI, respectively. All rabbits developed atherosclerosis and 60% had ruptured plaques. Rupture-prone plaques had higher vessel wall relaxation rate (R_1) (2.30±0.5s^{-1} vs. 1.86±0.3s^{-1}, *P*<0.001), measured 30min following gadofosveset administration, and higher R_1/plaque area ratio (0.70±0.06 vs. 0.47±0.02, *P*=0.01) compared with stable plaque at 12-weeks. Rupture-prone plaques had higher percent change in R_1 between the 3 and 12-weeks compared to stable plaque (50.80±7.2% vs. 14.22±2.2%, *P*<0.001). Immunohistochemistry revealed increased vessel wall albumin and microvessel density in diseased aortas and especially in ruptured plaque. Electron microscopy showed lack of structural integrity in both luminal and microvascular endothelium in diseased vessels. Functionally, the intrinsic vasodilation of the vessel wall decreased at 12-weeks compared to 3-weeks (18.60±1.0% vs. 23.43±0.8%, *P*<0.001) and in rupture-prone compared to stable lesions (16.40±2.0% vs. 21.63±1.2%, *P*<0.001). Arterial stiffness increased at 12-weeks compared to 3-weeks (5.00±0.1m/s vs. 2.53±0.2m/s, *P*<0.001) both in animals with stable and rupture-prone lesions.

Conclusions – T1 mapping using an albumin-binding contrast agent (gadofosveset) could quantify the changes in vascular permeability associated with atherosclerosis progression and rupture-prone plaques**.**

INTRODUCTION

Despite the successful implementation of several primary and secondary prevention strategies, atherosclerosis remains a crucial etiologic factor underlying stroke and myocardial infarction, which are the major causes of mortality and morbidity in Europe and the United States [1, 2]. Rupture of unstable atherosclerotic lesions is a major factor in the development of the thromboembolic sequelae following atherosclerosis and symptomatic disease [3, 4]. Damaged vascular endothelium and increased microvessel density leads to leakage of plasma components, including albumin, fibrinogen, erythrocytes, lipids, and inflammatory cells into the vessel wall and accelerate disease progression and lesion instability [4-7].

Vascular endothelium controls both vascular permeability, by regulating the flux of blood components through the vessel wall via intracellular and intercellular pathways [8], and vascular tone by balancing the production of vasodilators (e.g., nitric oxide, prostacyclin) and vasoconstrictors (e.g., endothelin-1, angiotensin II). However, cardiovascular risk factors impair both the structural integrity and functionality of endothelial cells, which renders the vessel wall pro-atherogenic by increasing endothelial permeability and attenuating endothelium-depended vasodilation [9, 10]. Additionally, plaque growth, local hypoxic conditions and inflammation stimulate microvessel growth within the plaque [4, 11]. Microvessel density correlates with the extent of the necrotic core, intraplaque haemorrhage [4, 12], and inflammatory infiltrates [13, 14] and is higher in ruptured compared with stable lesions in both the coronary [4, 15] and carotid arteries [4, 13-18]. Structurally, intraplaque and adventitial microvessels are immature, dysmorphic and fragile.

They lack surrounding α -actin-positive mural cells and are covered by structurally compromised endothelial cells, which makes them more leaky and permeable [11, 13, 14, 18-20].

Dynamic contrast enhanced (DCE) MRI using non-specific contrast agents e.g., Gd-DTPA has extensively been used to study plaque angiogenesis. Measurements of the contrast agent kinetic parameters, such as the transfer constant (K^{trans}) or the area under the signal intensity curve (AUC) showed correlation of contrast uptake with microvasculare density [21-24], adventitial vasa-vasorum [25, 26] and with sites of active inflammation [27] in both animal models and man. We [28-30] and others [22-24, 31] have used the albumin-binding MRI contrast agent, gadofosveset, to image vascular remodeling and treatment response in experimental and human atherosclerosis. Collectively, these studies show that gadofosveset uptake occurs through damaged endothelium and/or microvessels and may be a surrogate marker of vascular permeability. Gadofosveset is a clinically approved gadolinium-based agent. Approximately 70% of injected gadofosveset binds reversibly to plasma albumin that increases its r_1 relaxivity by 5-10 fold (r_1= 25 $mM^{-1}s^{-1}$) compared to the free fraction (r_1= 5 $mM^{-1}s^{-1}$) [32].

In this study, we used the MR albumin-binding contrast agent, gadofosvest, and T1 mapping in a rabbit model of atherosclerosis and controlled plaque rupture to investigated whether increased vascular permeability associates with plaque progression and rupture-prone plaque. This kind of non-invasive assessment of vascular leakage could provide both diagnostic and prognostic information for disease stratification.

METHODS

Animal Model

Ten New Zealand White (NZW) rabbits (Harlan, UK, 3 months old, male, 2.5 kg) were fed a 1% cholesterol-diet (Special Diet, Services, UK) for 2-weeks prior to and 6-weeks after aortic denudation. This was followed by 4-weeks of normal chow diet **(Figure 1; Supplemental Data)**

[33]. Aortic denudation was performed under general anesthesia with acepromazine (0.75 mg/kg IM), ketamine (35 mg/kg, IM), xylazine (5 mg/kg IM) and maintained with isofluorane 1-2%. A 3F Fogarty catheter was introduced in the aorta through a right femoral artery cutdown. The catheter was first advanced to the level of the diaphragm, inflated with saline and was retracted to the femoral artery. This procedure was repeated 3 times. Subsequently, the catheter was removed, and the incision closed.

At the end of the 12-weeks, diseased rabbits were injected with Russell's-viper-venom (0.15 mg/kg IP; Enzyme Research Laboratories, Swansea, UK), a procoagulant factor, followed 30min later by histamine, a vasoconstrictor in rabbits (0.02 mg/kg IV; Sigma-Aldrich, MO) to induce plaque rupture and thrombosis. This procedure was repeated twice within 4h. Four, non-balloon injured, age and gender matched rabbits (3 months old; male) were fed a normal diet for 12-weeks and used as controls. All procedures were approved by the United Kingdom Animal (Scientific Procedures) Act 1986.

In vivo MR Imaging

The abdominal aorta of NZW rabbits was imaged using a 3T Philips Achieva MR scanner (Philips Healthcare, Best, The Netherlands) and a 32-channel cardiac coil. Animals were imaged at 3 time points; 3-weeks and 12-weeks after initiation of the experimental protocol (pre-trigger scans) and 24h after the pharmacological triggering (post-trigger scan) **(Figure 1; Supplemental Data).**

The pre-trigger MRI included acquisition of native scout, phase-contrast MR angiography (PCA) and T1-weighted black-blood (T1wBB) images. The PCA images were acquired for visualization of the aorta, the renal branches, and the iliac bifurcation with: repetition time (TR) =20ms, echo time (TE)=3ms, flip angle (FA)=15°, FOV=300x150mm, acquired matrix=256x122, resolution=1.2x0.6x1mm, slices=20, Venc=150cm/s and averages=2. The maximum intensity projection (MIP) images were used to plan the subsequent scans. ECG-triggered 2D T1wBB

images were acquired with: TR/TE=2 heartbeats/5.4ms, profile order=centric, echo train length=6, BB delay=350ms, FOV=120x85mm, acquired matrix=384x270, resolution=0.31x0.31mm, slice thickness=4mm, slices=25 and averages=2. Subsequently, gadofosveset (0.03mmol/ kg, Ablavar, Lantheus Medical Imaging, North Billerica) was administered intravenously (IV). Immediately and up to 30 minutes after contrast injection, steady state free procession (SSFP) cine and quantitative phase contrast angiography (QFlow) images were acquired to measure functional parameters of the vessel wall. 2D retrospectively EGC triggered SSFP images were acquired with TR/TE=7.8/3.9ms, FA =60°, FOV=85x121mm, acquired matrix=172x241, resolution=0.5x0.5mm, slice thickness=5mm, slices=11, heart phases=18, averages=3. 3D ECG triggered fast gradient echo QFlow images were acquired with: TR/TE=17/10ms, FA =20°, FOV=85x121mm, acquired matrix=172x241, resolution=0.5x0.5mm, slice thickness=8mm, slices=1, heart phases=12, averages=2, $V_{encoding}$=150cm/s in the FH direction.

T1 mapping images were acquired 30min following gadofosveset administration using a Look-Locker based gradient-echo sequence that employs a non-selective inversion pulse with inversion times ranging from 20-2000ms, followed by eight segmented readouts for eight individual images for each of the two Look-Locker trains. In total, sixteen images per slice were acquired. T1 mapping parameters were: TR/TE=5.2/2.7ms, FA =10°, FOV=80x58mm, acquired matrix=200x141, resolution=0.4x0.4mm, slice thickness=4mm, slices=10, averages=1. Two stacks of T1 mapping sequences were acquired to cover a total length of 80mm in the FH direction.

Finally, the post-triggering MRI session was performed 24h after the pre-trigger scan and included acquisition of scout, PCA and native T1wBB images to visualize thrombus. Only the diseased rabbits underwent post-triggering MRI.

After the final MRI session, rabbits received heparin (1000 *USP* units IV, Sigma-Aldrich) to prevent post-mortem blood clotting and were sacrificed with a bolus injection of sodium

pentobarbital (100 mg/kg IV). The aortas were marked with suture ligature at distances above and below the left renal branch to match the total length imaged *in vivo*. The total length was measured with a ruler and the vessel was marked on its anterior site at every 2cm with tissue dye. After extraction, the ligatures were used to re-extend the aortas to their physiological length, with the ink marks used to stretch the vessel equally. Subsequently, the aorta of each animal was divided in segments that were allocated for different *ex vivo* studies. A total of 36 diseased vascular segments containing 21 stable and 15 ruptured plaque and 4 control segments were analyzed. Aortic segments allocated for light microscopy (n=18; 2 control; 10 stable and 6 ruptured plaques) and ICP studies [n=14; 2 control, 6 stable, 6 ruptured)] were snap frozen and stored in -80^{o}C Aortic segments used for electron microscopy [TEM n=8 (2 control, 3 stable and 3 ruptured), X-ray (n=2)] studies were fixed in 2% gluteraldehyde in 0.1 mol/L sodium phosphate buffer as described below.

MR Image analysis

Pre-trigger images acquired at 3 and 12-weeks were used to quantify plaque area, vascular permeability, aortic vasodilation and stiffness. The vessel wall was manually segmented by tracing the adventitial and the lumen contours on T1wBB images using the software OsiriX (OsiriX Foundation, Geneva, Switzerland). Plaque area (PA) was calculated as:

$$Plaque\ Area(PA) = Vessel\ Area - Lumen\ Area$$

Changes in plaque area between the 3 and 12-weeks were calculated using the T1wBB images based on the following formula:

$$\%\Delta T1wPA = \frac{\left(T1wPA_{12weeks} - T1wPA_{3weeks}\right)}{T1wPA_{3weeks}} * 100$$

T1 mapping images were used to calculate the vessel wall relaxation rate (R_1) on a pixel-by-pixel basis using a 3-parameter curve fitting procedure of the longitudinal magnetization including a T1 correction with an in house software (Matlab, Natick, MA) 30min after

administration of gadofosveset as previously published [34] and further explained in the Supplemental Data.

Changes in vascular permeability between the 3 and 12-weeks were calculated based on the following formula:

$$\%\Delta R_1 = \frac{\left(R_{1\ 12weeks} - R_{1\ 3weeks}\right)}{R_{1\ 3weeks}} * 100$$

Retrospectively ECG triggered SSFP images were used to calculate the intrinsic vasodilation of the vessel wall. The end-diastolic (ED) and end-systolic (ES) areas were manually segmented and the vasodilation of the aorta was calculated based on the formula:

$$\%Vasodilation = \frac{\left(ED - ES\right)}{ED} * 100$$

Changes in aortic vasodilation between the 3 and 12-weeks were calculated based on the following formula:

$$\%\Delta Vasodilation = \frac{\left(Vasodilation_{\ 12weeks} - Vasodilation_{\ 3weeks}\right)}{Vasodilation_{\ 3weeks}} * 100$$

Pulse-wave velocity (PWV) was used to characterize arterial stiffness. Qflow images were analysed using ViewForum (Philips Healthcare, Best, The Netherlands) and PWV was calculated based on the formula [35].

$$PWV(m/s) = \frac{\left(\Delta x\right)}{\left(\Delta t\right)}$$

where Δt=the time to peak velocity, Δx=distance between the proximal (immediately inferior to the left renal branch) and distal (immediately superior to the iliac bifurcation) slices of the imaging volume.

Changes in the PWV between the 3 and 12-weeks were calculated based on the following formula:

$$\%\Delta PWV = \frac{\left(PWV_{\ 12weeks} - PWV_{\ 3weeks}\right)}{PWV_{\ 3weeks}} * 100$$

Post-trigger images were used to identify the presence or absence of mural thrombus and to retrospectively classify the corresponding lesions, identified on the 12 weeks pre-trigger scan, in stable and rupture-prone, respectively [33, 36, 37].

Histology

Aortic segments (n=18; 2 control, 10 stable and 6 ruptured plaques) were transversely cryosections (10 μm) and stained with Masson's trichrome (Sigma Aldrich) to identify cellular components and thrombus. Ruptured plaques were defined as those with attached platelet and fibrin-rich thrombus whereas plaques without thrombus were defined as stable [33, 36]. The distances from the renal branches and the iliac bifurcation were used as internal anatomical markers to match the MR images and histological sections. Immunohistochemistry was carried out using biotinylated primary anti-human sheep polyclonal antibody for albumin (1:50, Abcam # ab8940, Cambridge, MA), and mouse monoclonal antibody for CD31 (1:50, DAKO JC70A, Cambridgeshire, UK) to locate the endothelium. Primary antibody binding was located using an avidin-peroxidase complex (Vector Laboratories, Burlingame, CA). Immunopositive areas were analyzed on digital images by computerized planimetry using ImageJ (National Institutes of Health). The immunopositive area (albumin) was segmented on the images and expressed as a percentage of the total plaque area. Plaque microvessels (immunopositive area for CD31) were counted individually and microvessel density was calculated by measuring the total number of microvessels and dividing by total plaque area.

Transmission Electron Microscopy (TEM)

The structural integrity of luminal and microvascular endothelium was studied with TEM. Aortic segments (n=8; 2 control, 3 stable and 3 ruptured) were fixed in 2% gluteraldehyde in 0.1 mol/L sodium phosphate buffer (pH 7.4) for 2h, washed with sodium phosphate buffer for 2h, and post-fixed in 1% OsO_4 for 2h. Samples were dehydrated through a graded series of ethanol and

embedded in epoxy resin. Semi-thin sections (0.2 μm) were stained with toluidine blue for light microscopy examinations and were used to guide sampling for transmission electron microscopy studies. Thin sections (0.09 μm) were collected on 150-mesh copper grids and double stained with uranyl acetate and lead citrate for electron microscopy examinations (H7650, Hitachi, Tokyo, Japan).

Electron Probe X-Ray Microanalysis

Aortic segments (n=2; stable plaque) were prepared by cryofixation under liquid nitrogen. Cryosections were cut at -120°C, transferred to Pioloform-coated nickel grids, and freeze-dried overnight. The sections were coated with a thin layer of carbon and viewed and analyzed in an FEI Tecnai 12 electron microscope equipped with an EDAX energy-dispersive x-ray spectroscopy detector. Mapping was achieved with EDAX software (EDAX) and used to display the distribution of gadolinium within the vessel wall.

Inductively coupled mass spectrometry (ICP–MS) for gadolinium quantification

To ensure the same pharmacokinetics/biodistribution of gadofosveset measured by T1 mapping *in vivo* and ICP-MS *ex vivo* the animals allocated for ICP-MS measurements were injected with gadofosveset after the post-trigger MRI and the agent was allowed to circulate in the body for 30min. The animals were then euthanized and aortas were collected as described above. For ICP-MS measurements aortic segments (n=14; 2 control, 6 stable, 6 ruptured) were digested in 70% nitric acid at 56^{o}C for 15-18h and diluted with deionized water for ICP-MS analysis. A standard curve was used for gadolinium concentration determination.

Statistics

The Statistical Package for the Social Sciences 21.0 (IBM Corporation, Somers, NY, USA) was used. For 2-group comparisons, continuous variables (e.g., control vs. diseased, control vs. stable/vulnerable, stable vs. vulnerable at the same time point) were compared using an unpaired t-test. However, a paired t- test was used to compare repeated measurements between 3- and 12-weeks (e.g., R_1 at 3 and 12 weeks for the same animal). Correlation analysis was performed using the Spearman test. Multiple segments from the same animal are assumed to be independent of each other. A second observer blinded to plaque status (stable/rupture-prone) re-analysed the R_1 maps to test the reproducibility of this measurement. The intraobserver and interobserver variability was assessed by using the interclass correlation coefficient (ICC) for continuous variables (R1 values). The ability of MRI variables to detect rupture-prone plaque was assessed using sensitivity, specificity, positive and negative predictive values (PPV and NPV), and receiver-operator characteristic curve (ROC) and its area under the curve (AUC). Logistic regression analysis was used to identify predictors of rupture-prone plaque with the presence/absence of thrombus as the outcome variable. Continuous data are presented as the mean±SEM**.** Probability values of $P<0.05$ were considered significant.

RESULTS

Vascular Permeability is Higher in Rupture-Prone compared to Stable Plaque

Consistent with previous studies, the 12-week pre-trigger MRI showed aortic atherosclerosis in all animals and the post-trigger MRI showed plaque rupture in 6/10 (60%) of the animals characterized by mural thrombus [33, 36, 37]. In a representative example, pre-trigger T1wBB images showed increased vessel wall area between 3 **(Figure 1A &1C**) and 12 weeks **(Figure 1E &1G)** (3.75±0.12 vs. 4.20± 0.18mm^2, P=0.04) indicating atherosclerosis progression**.** However, the plaque area was similar between stable and rupture-prone plaque at both the 3-weeks (3.82±0.13 vs. 3.71± 0.23mm^2, P=0.7) and 12-weeks (4.00±0.2 vs. 4.33±0.35mm^2,

P=0.58). The vessel wall R_1 relaxation rate, measured 30min after gadofosveset administration, was similar between stable and rupture-prone plaques at 3-weeks **(Figure 1B & 1D)** (1.50±0.12 s^{-1} vs 1.70±0.2s^{-1}). Conversely, at 12-weeks, the rupture-prone plaque had significantly higher R_1 relaxation rate compared to the stable plaque, indicating higher vascular permeability (1.83±0.25 s^{-1} vs 2.54±0.3s^{-1}) **(Figure 1F & 1H)**. Post-trigger T1wBB images **(Figure 1I & 1K)** and corresponding histology **(Figure 1J & 1L)** show absence of thrombus in the case of the stable plaque and presence of thrombus overlying the ruptured plaque. An additional example demonstrating R_1 and T_1 maps, both in gray-scale (B, G, D & I) and color-coded formats (C, H, E, & J), in visualizing and segmenting the vessel wall to calculate the vessel wall T_1/R_1 values is illustrated in **(Figure 2; Supplemental Data).** Interclass correlation coefficient (ICC) analysis revealed high intra-rater agreement (ICC, 0.89; 95% confidence interval [CI], 0.83-0.93, *P*<0.001) and good inter-rater variability (ICC=0.75, 95% CI=0.4-0.79, *P*<0.001) for the measurement of vessel wall relaxation rate R_1. The results of the blinded observer are illustrated in Supplemental Table 1, along side observer one**.**

Quantitative MRI Measurements

Quantification of vascular permeability to gadofosveset (R_1) during atherosclerosis progression in the rabbit aorta is illustrated in **Figure 2**. The vessel wall R_1 was similar in control and diseased animals at 3-weeks (1.69±0.02s^{-1} vs. 1.71±0.2s^{-1}, *P*=0.19) but was significantly higher at 12-weeks (1.71±0.2s^{-1} vs. 2.00±0.1s^{-1}, *P*<0.001) **(Figure 2A)**. When the vascular segments were classified in stable and rupture-prone, the vessel wall R_1 was similar between the groups at 3-weeks (1.70±0.17s^{-1} vs. 1.75±0.6s^{-1}, *P*=0.33). However, at 12-weeks, rupture-prone plaques had significantly higher gadofosveset uptake resulting in higher R_1 values compared to stable plaque (2.30±0.5s^{1} vs.1.86±0.3s^{-1}, *P*<0.001). Paired t- test analysis showed a significant increase of R_1 between 3- and 12-weeks for both the stable (1.70±0.17 vs. 1.86±0.3, *P*<0.001) and rupture-prone lesions (1.75±0.6 vs. 2.30±0.5, *P*<0.001) **(Figure 2B)**. To account for

changes in R_1 that might be affected merely by lesion size the R_1 was normalized to plaque area, as measured by T1wBB images. The R_1/ plaque area ratio was higher in rupture-prone compared to stable plaque (0.70±0.06 vs. 0.47±0.02, *P*=0.01) at 12 weeks **(Figure 2C)**. Paired t-test analysis showed a small but significant increase in the R_1/ plaque area ratio between 3- and 12-weeks for rupture-prone lesions (0.55±0.04 vs. 0.70±0.06, *P*=0.04). The percentage increase in R_1 between 3 and 12-weeks was higher for rupture-prone compared to stable plaque (50.80±7.2% vs. 14.22±2.2%, *P*<0.001) **(Figure 2D)**. Gadolinium concentration was higher in diseased compared to control animals (50.45±9.2 vs. 22.13±1.0 μg/gr tissue, *P*=0.03) and rupture-prone compared to stable plaque (80.54±6.13 vs. ± 32.40±4.0 μg/gr tissue, *P*<0.001) as calculated by ICP-MS.

Vascular Permeability to Albumin is a Predictor of Plaque Rupture

The value of the quantitative assessment of vascular permeability (R_1) after administration of gadofosveset in detecting rupture-prone plaque was analyzed with ROC curves at 12-weeks **(Figure 3A-3C)**. ROC curves showed that R_1, R_1 / plaque area and the $\% \Delta R_1$ between 3 and 12-weeks had high areas under the curve and were good predictors of rupture-prone **(Table 1)**. Logistic regression analysis showed that the R_1 at 12wks is a predictor of rupture-prone plaque (*P*<0.001, OR=11.45, CI = 3.89-33.72).

Histological analysis shows higher Intra-Vessel Wall Albumin and Microvessel Density in Ruptured compared to Stable Plaque

Quantification of the albumin-immunopositive areas showed a significant increase in intra-vessel wall albumin in diseased compared to control vessels **(Figure 4A-4C;** brown color and **4D)** (33.00±3.3% vs. 12.91±0.8%, *P*=0.01) and particularly in ruptured compared to stable plaque (44.84±4.0%vs. 25.93±3.0%, *P*=0.03) **(Figure 4E)**. CD31-staining of the endothelium (Figure 4F-4H; brown color) showed intact, continuous endothelial cells in control vessel, less structured

cells in stable plaque, and fragmented cells covering the lumen of ruptured plaque. Endothelium staining also showed higher microvessel density in ruptured compared to stable plaque (21.00±2.24 vs 7.32±0.96, *P*<0.001) and absence of microvessels in control arteries (**Figure 4I-4K; asterisks and 4L**).

Transmission Electron Microscopy (TEM) shows abnormal morphology and aberrant junctions of luminal and microvascular endothelial cells

TEM was used to examine the ultrastructural integrity of luminal and microvascular endothelial cells. Control luminal endothelium was characterized by intact basal lamina (BL) and inter-endothelial junctions indicated by close contacts **(Figure 5A)**. However, in diseased arteries endothelial integrity was severely compromised. The luminal endothelium appeared detached from the basal lamina **(Figure 5B)** with numerous intra-cytoplasmic vacuoles **(Figure 5C)**. The inter-endothelial contact was incomplete or completely absent allowing infiltration of blood erythrocytes within the vessel wall **(Figure 5C)**. In more advanced cases, the luminal endothelium showed signs of cellular death including lack of nucleus and denudated regions allowing exposure of the subendothelial space **(Figure 5D)**. Similarly to the luminal endothelium, microvascular endothelium was also structurally compromised **(Figures 5E-5H)**. In some cases, microvascular endothelial cells showed intact BL and closed inter-endothelial junctions **(Figure 5E)**. However in other cases, microvascular endothelial cells showed various structural changes **(Figures 5F-5H)**. Although an intact basal lamina was generally observed, inter-endothelial contact was incomplete **(Figure 5G; dashed allows)** and endothelium morphology was changed to a more cuboidal-shape with numerous intra-cytoplasmic vacuoles **(Figure 5G; arrows)**. Detachment of endothelial cells from the BL and leucocytes adhering to the microvessel lumen and infiltrating into the plaque were also observed **(Figure 5H)**.

TEM and gadolinium mapping using x-ray spectra showed a diffuse distribution of gadofosveset across the vessel wall and lack of co-localization to particular plaque or vessel wall components **(Figure 3; Supplemental Data).**

There were significant correlations between vascular permeability (measured by R_1) and plaque area (measured by T1wBB) at 3 (*r*=0.23, *P*=0.03) and 12-weeks (*r*=0.24, *P*=0.03) **(Figure 4A and 4B; Supplemental Data)**. However, when the analysis was repeated with vascular segments classified as stable or rupture-prone the correlation was more significant for stable (*r*=0.4, *P*=0.004) but disappear for rupture-prone (*r*=0.2, *P*=0.44) lesions at 12-weeks **(Figure 4C and 4D; Supplemental Data)**. We also found that the percentage increase in R_1 was not followed by an equivalent increase in plaque area **(Figure 5A; Supplemental Data)**. The percentage increase in vascular permeability ($\%\Delta R_1$) to gadofosveset between 3 and 12-weeks was significantly higher for rupture-prone compared to stable plaque (50.80±7.2% vs. 14.22±2.2%, *P*<0.001). However, the percentage increase of plaque area (% Δ T1wPA) was not statistically different between the two lesion types (35.00±3.5% vs. 41.00±9.0%, *P*=0.2)**.** Furthermore, there was lack of correlation between the percentage change of plaque area (% Δ T1wPA) and the percentage change in vascular permeability ($\%\Delta R_1$) between 3 and 12-weeks **(Figure 5B; Supplemental Data**) when all lesions were included (*r*=0.16, *P*= 0.8). However, there was a significant correlation between % Δ T1wPA and $\%\Delta R_1$ for stable (*r*=0.32, *P*=0.02), but not for rupture-prone lesions (*r*=-0.08, *P*=0.48) **(Figure 5C and 5D; Supplemental Data**).

Intrinsic Vascular Elasticity Decreases in Diseased Vessels and is lower in Rupture-Prone compared to Stable Plaque

Functional changes in the intrinsic vasodilation and wall stiffness were also investigated. The intrinsic vasodilation was lower in atherosclerotic compared to control animals (control=26.80±1.72% vs 3-weeks=23.43±0.8%, *P*=0.07, control vs 12-weeks =18.60±1.0%,

P<0.001) **(Figure 6A).** Vasodilation was even lower in rupture-prone compared to stable lesions at both the 3-weeks (21.21±1.3% vs. 24.41±1.13%, *P*=0.02) and 12-weeks time points (16.40±2.0% vs. 21.63±1.2%, *P*<0.001) **(Figure 6B)**. However, there was lack of correlation between the intrinsic vasodilation and plaque area at both the 3 (*r*=-0.02, *P*=0.45) and 12-weeks (*r*=-0.02, *P*=0.5; data not shown).

Aortic stiffness measured with PWV increased in diseased aortas at 12-weeks compared to 3-weeks and control animals (5.00±0.1m/s, 2.50±0.2m/s, 2.53±0.2m/s) **(Figure 6C)**. Paired t-test showed a significant increase in PWV between 3- and 12-weeks in both animals with stable (2.49±0.3m/s vs. 5.23±0.4, *P*=0.03) and animals with rupture-prone (2.49±0.1m/s vs. 5.00±0.04, *P*<0.001) lesions. However, PWV was not statistically different between animals with stable and rupture-prone plaques at each time point (2.49±0.3m/s vs. 2.49±0.1m/s, *P*=1.0 and 5.23±0.35m/s vs. 5.00±0.03m/s, *P*=0.55) **(Figure 6D)**. There was no correlation between PWV and plaque area at 3-weeks (*r*=0.54, *P*=0.2) but there was a significant correlation at 12-weeks (*r*=0.9, *P*=0.037) **(Figure 6E)** suggesting that lesion expansion increases arterial stiffening. Finally, there was a significant negative correlation between the percentage changes in the intrinsic vasodilation and arterial stiffness (*r*=-0.96, *P*=0.003) **(Figure 6F)**.

CONCLUSIONS

This study demonstrates that vascular permeability, measured using an MR albumin-binding contrast agent and T1 mapping, is elevated in atherosclerotic compared to control vessels and, more importantly, is higher in rupture-prone compared to stable atherosclerotic lesions in a rabbit model of the disease. Increased vascular permeability measured *in vivo* was histologically validated by increased intra-vessel wall albumin, higher microvessel density and lack of structural integrity of both the luminal and microvascular endothelium. Functionally, the intrinsic capacity of the vessel wall to vasodilate was significantly lower in diseased, particularly, in rupture-prone lesions. Furthermore, pulse wave velocity analysis revealed significantly

increased arterial stiffness in atherosclerotic compared with control animals, but similar levels of stiffness between animals that had stable and rupture-prone lesions. We showed that MRI can quantify both morphological and functional changes of the vessel wall and that non-invasive quantification of vascular permeability using an albumin-binding MR contrast agent may have diagnostic value in detecting not only plaque progression but also unstable atherosclerotic lesions.

Traditionally, "bright-blood" DCE-MRI has been used to study plaque permeability. Measurements demonstrated that the kinetic parameters including fractional plasma volume (vp) and K^{trans} (expressing flow/permeability) correlate with microvessel density [21-24], adventitial vaso-vasora [25, 26] and with sites of active inflammation [27]. K^{trans} was also used to monitor response to therapeutic intervention [38]. Human subjects treated with lipid lowering therapy for one year showed reduced K^{trans} independently of a reduction in the lipid-rich necrotic core size or circulating levels of C-reactive protein [38]. Despite the promising applications of "bright blood" DCE-MRI, acquisitions are usually suited for the characterization of lesions thicker than 2mm, because of the close proximity of the enhancing vessel lumen, which makes vessel wall segmentation challenging. Alternatively, contrast uptake can be evaluated using "black-blood" DCE-MRI by measuring the area under the curve (AUC). Experiments in atherosclerotic rabbits showed a correlation between AUC, after administration of Gd-DTPA, and histological markers of plaque neovascularization [21].

Our findings using a T1 mapping approach showed that the vessel wall relaxation rate (R_1) after gadofosveset administration could also be used to quantify changes in vascular permeability in the context of atherosclerosis. We found that vessel wall permeability, measured as R_1, was significantly higher at 12 compared to 3-weeks after commencing the experimental protocol and control animals. Importantly, rupture-prone plaques had significantly higher R_1 values and R_1/ plaque area ratio compared to stable lesions at 12-weeks. The percent change in R_1 between 3 and 12-weeks was also significantly higher for rupture-prone compared to stable

lesions. We have previously shown that vessel wall uptake of the albumin-binding contrast agent occurred through the progressive loss of luminal endothelial cell integrity in a murine model of atherosclerosis that lacked angiogenesis within the timeframe of the study [28]. We also showed that treatments aiming to restore endothelial cell integrity decrease vascular permeability to albumin and retard plaque development [28, 29]. Other studies have demonstrated that uptake of gadofosveset occurs through a combination of damaged endothelium and/or microvessels in experimental atherosclerosis [24, 28, 31]. In our current study, we found that leakage of the albumin-binding contrast agent associates not only with plaque progression, as we have previously shown [28, 29], but also with lesion instability using a rabbit model that replicates multiple histological features described for human plaques [33, 36, 37]. The ability to experimentally induce plaque rupture and thrombosis at a precise time point provides a functional end-point to retrospectively classify plaque as non-ruptured/ stable (no thrombus) or ruptured/vulnerable (mural thrombus). Therefore, this study protocol allows determination of imaging measurements that could potentially "predict" complication that cannot be achieved in the murine models we have previously used. The clinical utility of gadofosveset for vessel wall imaging was shown in a small proof-of-principle study that showed significantly higher gadofosveset uptake in symptomatic compared with asymptomatic human carotid plaques [23]. Our current results are in line with human data [23] and pinpoint the potential value of quantitative measurement of vascular permeability using the albumin-binding contrast agent for identification of high-risk/ unstable plaques.

Histologically we found increased intra-vessel wall albumin [23, 24] and microvessel density in diseased, and in rupture plaques in particular. TEM analysis revealed loss of the endothelial integrity with disruption of inter-endothelial junctions in both the luminal and microvascular endothelium of atherosclerotic plaques. Both luminal and miscrovascular endothelial cells appeared detached from the basal lamina, had a cuboidal shape and numerous intra-cytoplasmic vacuoles, a sign of increased secretory capacity. Inter-endothelial contact was

incomplete or absent allowing infiltration of blood cells (erythrocytes, leucocytes) into the vessel wall. Studies in humans showed that rupture-prone and ruptured coronary plaques exhibit a 2-fold and 4-fold increase in microvessel density, respectively, compared to stable obstructive plaques [4, 15] Similarly, ruptured carotid endarterectomy [19, 20] and aortic [7] plaques had increased microvessel density, with larger, immature, more irregular-shaped microvessels comparable to those found in tumors and healing wounds. Thin-walled microvessels showing incomplete endothelial junctions and lack of structural integrity have also been reported in human coronary plaques and were used to explain the extensive leukocyte infiltration, intraplaque hemorrhage, and plaque instability [11]. Our histological data corroborate previous observations and suggest that increased microvessel density and compromised structural integrity (of both the luminal and microvascular endothelium) increase vascular leakage that facilitates extravasation of inflammatory cells and molecules that may contribute to plaque instability. Our *in vivo* measurements further validate *ex vivo* histological findings and provide a quantitative method for *in situ* measurement of focal vascular leakage associated with atherosclerosis progression and plaque instability using the albumin-binding contrast agent as a surrogate marker.

Our study also revealed some *in vivo* mechanistic insights on the role of vascular leakage in the natural evolution of atherosclerosis and plaque instability. Although we found that vascular permeability was increased in diseased compared to control arteries and in rupture-prone compared to stable lesions, the percentage increase in vascular permeability ($\% \Delta R_1$) was not followed by an equivalent increase in plaque area ($\% \Delta$ T1wPA) between 3 and 12-weeks when all arterial segments were analysed together. However, when stable and rupture-prone lesions were analysed independently, a significant correlation between $\% \Delta$ T1wPA and $\% \Delta R_1$ was found for stable lesions. These data suggest that the rate of plaque growth maybe affected by changes in vascular permeability at earlier stages of disease progression. We speculate that at some point during plaque evolution increased vascular leakage because of *i)* increased

microvessel density, *ii)* differential permeability of individual microvessels, or *iii)* lack of structural integrity of the luminal or microvascular endothelium precedes plaque expansion causing a divergence from linearity between the increase of vascular permeability and focal lesion. To this end, measuring vascular permeability may provide a more sensitive biomarker for assessing atherosclerotic risk.

This study has also revealed functional changes (vasodilation and stiffness) in the vessel wall in atherosclerosis, in particular in relation to the properties of stable compared with rupture-prone plaque. Intrinsic vasodilation decreased with the progression of atherosclerosis and was lower in rupture-prone compared with stable lesions. However, there was no significant correlation between the intrinsic vasodilation and plaque area suggesting that factors other than plaque size may contribute to the reduced vasodilation. Other studies have shown that structural changes in the vascular endothelium reduce endothelium-dependent vasodilation in response to endothelium stressors [9, 10] or mechanoreceptor signaling [39, 40]. Endothelial cell (EC) surfaces have multiple mechanoreceptors that sense and respond to changes in endothelial shear stress (ESS) via intracellular mechanotransduction pathways. Physiologic pulsatile ESS regulates continuous nitric oxide (NO) production by the endothelium either at the transcriptional level, through upregulation of endothelial nitric oxide synthase (eNOS) gene expression [41], or at the post- transcriptional level by eNOS protein phosphorylation and activation [42]. NO is a potent regulator of healthy vascular tone [43]. Low ESS reduces the bioavailability of NO by decreasing eNOS messenger ribonucleic acid (mRNA) and protein expression, down-regulates prostacyclin, an endothelial vasodilatory substance [44] and upregulates endothelin-1 (ET-1) [44], a vasoconstrictive molecule. Although we did not measure ESS in this study, our previous work using this animal model demonstrated that low ESS was associated with plaque burden, positive vascular remodeling, and plaque instability [45]. Similar results were shown using other animal models and in humans [46] and could probably explain the reduction in the intrinsic vasodilation. Our study also showed coupling between morphological (plaque area) and functional (stiffness)

vascular changes. PWV did not increase in the early stages of atherosclerosis (3-weeks), but increased after 12-weeks and correlated with plaque area. However, the increase in PWV was similar in animals with and without rupture-prone plaques. Our data suggest that vascular stiffening maybe a measure of overall vascular health (when atherosclerotic lesions are well formed), but it is not a surrogate marker of the propensity of a plaque to rupture in this animal model. Similar data have been reported in human studies where PWV was shown to increase with age [47], in the presence of calcification [48], hypertension [49] and intraplaque hemorrhage [50].

STUDY LIMITATIONS

A potential limitation of our study is that non-contrast T1wBB (0.31x0.31mm) had a higher in-plane resolution compared with the T1 mapping (0.4x0.4mm) and that partial volume effects and motion might influence the accuracy of the R_1 measurements. However, the thickness of the normal vessel wall in the rabbit aorta is ~0.5-0.6mm and therefore the in-plane resolution should have been sufficient to delineate the vessel wall on both sequences.

The higher ICC observed for the intra-reader variability compared to the inter-reader variability is not surprising and suggested a higher variability between independent observers when manually segmenting the vessel wall for measuring the R1 when compared to the same observer. Manual vessel wall segmentation is known to be both time consuming and also subject to observer variability. In our study, the R1/T1 maps were analysed using a dedicated Matlab code that could only load the T1 mapping datasets. Fusion of the R_1/T_1 maps with the corresponding black-blood images that could potentially facilitate vessel wall segmentation was not possible. Future, adaptations of our code might allow for this function. In addition, the future use of 3D MOLLI- based T1 mapping sequences that allow for immediate reconstruction of the T1/R1 maps that can be directly imported on the DICOM viewer along with the black-blood images, perform image fusion and co-registration might also improve the accuracy and decrease inter-rater the variability in vessel wall segmentation.

CONCLUSIONS

Compromised structural integrity of the luminal and microvascular endothelium and higher microvessel density increase vascular permeability in atherosclerosis and particularly rupture-prone plaque. T1 mapping using an albumin-binding contrast agent could be used to quantify changes of vascular permeability associated with atherosclerosis and plaque instability**.**

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CONFLICT OF INTEREST

None

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TABLES

Table 1: Ability of MRI measurements in detecting rupture-prone plaques

	$R_1 \geq 1.9\ s^{-1}$ and % (CI)	R_1/T1wPA $\geq 0.3938\ (s^{-1}/mm^2)$ and % (CI)	$\Delta R_1 \geq 24.81\%$ and % (CI)
Sensitivity	81.2 (63.8-92.1)	90.6 (73.8-97.5)	65.6 (48.1-81.4)
Specificity	72.5 (58.0-83.6)	45.0 (91.8-59.5)	84.3 (71.4-92.9)
PPV	48.2 (37.2-59.3)	68.7 (57.4-78.2)	72.4 (52.8-87.3)
NPV	51.8 (40.0-62.8)	31.3 (21.8-42.6)	79.6 (66.5-89.4)

FIGURE CAPTIONS

Figure 1: *In vivo* MRI shows higher vascular permeability in rupture-prone compared to stable plaque in rabbit atherosclerosis.

Native pre-trigger T1wBB images acquired at 3 **(A, C)** and 12-weeks **(E, G)** show increased vessel wall thickening due to plaque formation. Corresponding R_1 relaxation maps (**B, D, F, H)** shows higher vessel wall relaxation rate (R_1)(orange/yellow color; arrows) in rupture-prone **(H)** compared with the stable plaque **(F)** at 12-weeks indicating higher vascular leakage to gadofosveset. Post-trigger T1wBB images **(I, K)** and histology **(J, L)** show the absence of thrombus at the site of a stable plaque and the presence of a thrombus overlying the ruptured plaque. Ao: aorta, V: vein. N=10 rabbits, 6 with ruptured and 4 without ruptured lesions were included in the MRI analyses.

Figure 2: Quantification of MRI measurements after administration of gadofosveset.

(A-B) Vessel wall R_1 increased with disease progression and is higher in rupture-prone compared to stable plaque at 12-weeks**. (C)** R_1/ plaque area was higher in rupture-prone compared to stable plaque at 12-weeks**. (D)** The percentage change in vessel wall R_1 between 3

and 12-weeks was significantly higher in rupture-prone compared to stable plaque. N=10 rabbits, 6 with ruptured and 4 without ruptured lesions were included in the MRI analyses

Figure 3: Vascular permeability to gadofosveset is a predictor of plaque-prone plaques. Receiver-operator characteristic curve at 12-weeks showed that R_1 **(A)**, R_1/ plaque area and **(B),** and the percentage ΔR_1 **(C)** from 3 to 12-weeks have high areas under the curve and are good predictors of plaque instability. N=10 rabbits, 6 with ruptured and 4 without ruptured lesions were included.

Figure 4: Immunohistochemistry of rabbit atherosclerotic plaques.
Albumin immunohistochemistry **(A-C)** and quantification of the albumin immunopositive area **(D-E)** showed accumulation of albumin (brown color) in atherosclerotic vessel and particularly in ruptured compared to stable lesions. Endothelium immunohistochemistry staining (brown color) shows fragmented cells in ruptured **(H)** compared to stable plaque **(G)** and control tissue **(F)**. Endothelium immunohistochemistry shows absence of microvessels in control tissue **(I)** and abundant microvessels (asterisks, *****) in ruptured **(K)** compared to stable plaque **(J)**. Microvessel density was higher in ruptured compared to stable plaque and absent in control vessels **(L)**. P=plaque and M=media. N=18; 2 control, 10 stable and 6 ruptured plaques were included.

Figure 5: Transmission electron microscopy of the structure of the endothelium.
Images of luminal endothelial cells in control **(A)** and diseased aorta **(B-D).** The endothelium in control arteries had intact basal lamina and inter-endothelial junctions indicated by close contact. In diseased aortas, the endothelial integrity was compromised and the endothelium appeared detached from the basal lamina **(B)** with numerous intra-cytoplasmic vacuoles **(C)**. The inter-endothelial contact was incomplete or completely absent allowing infiltration of blood erythrocytes within the vessel wall **(C)**. The luminal endothelium showed signs of overt cellular

death including lack of nucleus and denudated regions allowing exposure of the subendothelial space **(D)**. Images of microvascular endothelial cells **(E-H)**. Some microvascular endothelial cells showed intact basal lamina and closed inter-endothelial junctions **(E)**. In other cases, although an intact basal lamina was observed **(F)**, inter-endothelial contact was incomplete in plaque microvessels **(G; dashed allows)** and endothelial cell morphology was changed to a more cuboidal-shape with numerous intra-cytoplasmic vacuoles **(G; arrows)**. Detachment of endothelial cells from the basal lamina, endothelial death and leucocytes adhering to the microvessel lumen thus infiltrating the plaque were also observed **(H)**. BL: basal lamina, EC: endothelial cell, RBC: red blood cell. N=8; 2 control, 3 stable and 3 ruptured were included.

Figure 6: Quantification of the Intrinsic Vasodilation and Arterial Stiffness in rabbits by MRI. The intrinsic vasodilation decreased in diseased compared to control aortas **(A)** and even more in rupture-prone compared to stable lesions **(B)**. Pulse wave velocity indicative of vascular stiffness increases in diseased compared to control **(C)** animals but it is similar between animals with and without rupture-prone plaques **(D)**. There was a significant correlation between plaque area and arterial stiffness at 12-weeks **(E)**. There was a significant negative correlation between the percentage change of the intrinsic vasodilation and the pecentage change of the PWV between the 3 and 12-weeks **(F)**. N=10 rabbits, 6 with ruptured and 4 without ruptured lesions were included.